A STUDY OF THE GROWTH OF PASTEURELLA AVICIDA

by

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I. INTRODUCTION

In the course of a study of fowl cholera we became interested in the growth of Pasteurella avioida, the causative organism, in the body of the infected bird. The disease produced by this organism is considered to be a true septicemia and does illustrate that type of infection as well as any organism.

Very little work has been done to determine how the organism develops in the body. Some of the earlier writers state that it grows rapidly in the warm cadaver, but do not report on growth curves during life. Others are inclined to confer little pathogenic significance on this organism and believe that it may be an invader of the tissue and blood only following death.

This report is on the development of the organism, Pasteurella avioida in the body of the susceptible birds. We have not been able to immunize birds to a point of strong immunity against the organism when injected intravenously in heavy doses, so we cannot report on the growth in immune birds.

II. REVIEW OF LITERATURE

Bull (1) found that typhoid bacilli injected into the blood of normal rabbits were agglutinated and phagocytized so that they disappeared very quickly from the

blood stream. He found that in twenty minutes after injection of large numbers of a virulent culture the blood became free from bacteria. An examination of the crushed organs at the time of disappearance from the blood stream of the animal showed large numbers in the tissue spaces but not enough to account for all the bacteria injected.

This same writer (2) also found that pneumococci circulating in the blood stream were agglutinated very rapidly and completely following the injection of an immune serum. This action was specific but its nature was not explained. Agglutination in the body took place in much smaller dilutions of serum than was effective in the test tube. He found that the Shiga type of dysentary bacillus was not agglutinated in twenty minutes, while the Flexner type was rapidly agglutinated. When the animal was killed within a few minutes leucocytes carrying large numbers of the Flexner type were found in the lungs, liver and spleen of the animals. A non-virulent strain of the influenza bacillus was rapidly agglutinated while a virulent strain was not. When these organisms are not promptly removed from the blood stream, they grow and produce a fatal septicemia.

The same means which causes clumping and removal of bacteria from the blood and their accumulation in the organs, also causes a leucopenia with accumulation of poly-

morphonuclear leucocytes in the same organs. The diplococci which fail to be agglutinated tend not to be phagocyted and persist longer in viable form than these in the clumps.

In a discussion of the immunity factors in pneumococcus infection in the dog, Bull (4) points out that there is a very rapid decrease in the bacteria injected, followed in forty-eight to ninety-six hours by a slight increase. He considered the time lapse between the first decrease and the rise, as a time necessary for the organisms to adapt themselves to a new and adverse environment. When they once become immune to the injurious antibodies present a rapid multiplication occurred and symptoms of disease became manifest. If these facts can be used to interpret the incubation period of infectious disease in man, the logical conclusion is that it has a similar meaning in the dog, for if bacteria find ideal conditions for multiplication on entering the new host, only a few hours will elapse between the time of infection and the appearance of symptoms of disease. In the pneumococcous septicemia in rabbits the result is fatal before the defensive antibodies can be formed, while in dogs, the second decrease is due to antibodies which are formed after infection.

Bull (5) also reported a study of Bacillus avisep-

tions in dogs and rabbits. He considered the organisms as highly toxic to both animals and found 1 cc. per kilo. to be sufficient to cause death.

Hadley (6) later refuted these findings and determined that Bull was using a culture of the organisms of fowl typhoid.

Traube and Gscheidlen (7) conducted the first experimental work on the development of organisms in the blood of warm blooded animals. They injected rabbits and dogs intravenously with mixtures of putrefactive bacteria. They found that blood removed from these animals under aseptic conditions did not putrify and considered the ozonized oxygen of the blood corpuscles was harmful to the bacteria. If very large masses of bacteria were injected the animal died in 24 to 48 hours. Then they found bacteria in the blood a short time before death.

Cheyne (8) found that when he injected one-fourth to three-fourths of a cc. of bacterial culture into healthy animals, the animals survived, but if they were poisoned with phosphorous or injected with large amounts of culture the animal succumbed.

Fodor (9) injected twenty to fifty million bacteria into rabbits and examined their blood at various intervals. He found that in the healthy animals the organ-

ism disappeared from the blood in four to eight hours, but were much slower in disappearing from the blood of starved or weak animals.

Wyssokowitsch (10) made a study of the injection of pure cultures of organisms grown on solid media and suspended in salt solution. He studied both saprophytic and pathogenic bacteria and molds.

Berkman, Nelson and Palmer (11) used guinea pigs lacking vitamin C which were scorbutic. They used pneumococci and B. anthracis as their infecting material. Guinea pigs suffering from the lack of vitamin C experienced a definite and determinable, though not marked, break in their resistance to these organisms. The reduced body temperature is of primary significance in accounting for the reduced resistance to infection. Guinea pigs lacking vitamin C reveal no difference in their ability to produce specific agglutinins for the typhoid bacillus from that of healthy animals. Investigations of the phagocytic activities in guinea pigs lacking vitamin C reveal no injury to the phagocytic mechanism as the result of vitamin C deprivation.

Smith (12) states that reducing the intake of vitamin A to a minimum level which is just compatible with life decreases the resistance of the normal rat to tuberculo-protein but slightly, whereas the susceptibility of

the tubercle bacillus infected rat is so greatly increased that it generally succumbs to very small amounts of tuberculo-protein.

III. NATURE OF THE DISEASE

1. Type of Infection

Fowl cholera is an acute infectious septicemia of domesticated and wild birds. The portal of entry is probably the mucous membranes of the pharynx or nasal cleft. The birds usually die within one to three days after infection, depending on the virulence of the organism and bodily resistance.

2. Growth of Organisms in the Body of Bird

The growth of the organism in the body of the bird is still open to much discussion. The only method available is a study of the organs of the bird after death has occurred. This leaves for discussion the rate of development in the organs, the possibility of mechanical removal from the blood stream and the rate of development in the blood stream. These points are discussed more in detail later in this paper.

3. Post-mortem Changes in Acute Cases

Lesions found on post-mortem include petechial hemorrhages on the heart and visceral fat, congestion of the lungs, liver, spleen and intestinal mucosa, and enormous numbers of organisms free in the blood stream. Sections of the organ tissues show the organisms present in the mucous exudate of the alveoli and bronchi of the lungs. In these areas there appears to be large colony like masses of bacteria. Some of these appear to be of different types although on culture they seem to be the same organism. The organisms are found to a lesser extent in the spleen, liver, kidneys, and muscles.

4. The Cause of Death

The exact cause of death in fowl cholera is as yet a debatable question. The most likely explanation is that death is a result of sub-oxidation of the body tissues. The cause of this sub-oxidation is not known. The lack of oxygen may be due to competition between the body cells and the enormous number of bacteria free in the blood stream; again it may be due to the decreased lung capacity caused by the collection of bacterial clumps and exudate in alveoli and bronchi; or both the

above causes.

IV. EXPERIMENTAL

The phase which seemed to offer the best solution to the above problem was to study the rate of growth and total numbers of bacteria present in the blood of the infected birds during life. The problem was approached from this angle. Several methods of artificial inoculation were considered. The method of intravenous injection was chosen for the following reasons:

(1). The time of entrance of the organism into the blood stream is definitely known.

(2). The number of bacteria entering the blood stream at the beginning of infection is known.

(3). The protective power of body may be studied directly.

(4). This method of infection is easy to accomplish.

(5). Birds are in practically normal condition at the time the organism enters the blood stream.

1. Cultures Used

Cultures Nos. P 16-978 and 4777 of proved purity and pathogenicity were used throughout this work. Culture 4777 was isolated from a mature bird from a field

case of fowl cholera. Culture P 16-978 was isolated from a ten day old chick. Both cultures were typical of Pasteurella avicida in carbohydrate fermentation and in staining characteristics. Dr. Beaudette of the New Jersey Agricultural Experiment Station described culture P 16-978 as follows. "A typical fluorescent type. In tests it killed young chicks in 48 hours when instilled into the nasal cavity. When ten adult birds were similarly treated, four died."

Cultures for inoculation were grown in chicken meat-infusion broth. This broth was prepared in the following manner:- Fresh chicken meat was run through a meat grinder and added to distilled water in the ratio of 500 grams of meat to 1,000 cc. of water. To this was added 0.2 percent sodium citrate. This mixture was stirred well and placed in the ice box over night. It was then autoclaved at 20 pounds pressure for 60 minutes. The broth was then removed by straining through one thickness of cheesecloth. To this stock broth was added 2 percent peptone (Parke-Davis Bacteriologic) and 1 percent C.P. sodium chloride. The reaction was adjusted to pH 7.2. The media was then tubed and sterilized at 20 pounds of pressure for 30 minutes.

The broth tubes were inoculated from a 24 hour growth of Pasteurella avicida on hemolyzed blood agar.

This hemolysed blood agar will be described later. These cultures were then incubated 19 to 24 hours at 37°C. These cultures furnished the supply of inoculating material for each days work. As it was impossible to inoculate and study but one bird each day, cultures as nearly of uniform composition and age were used throughout the work.

In preparing the inoculant many precautions were necessary. As the Pasteurella avicida organism grows with a typical slimy growth, which settles to the bottom of the tube in 15 to 20 hours, only the top half of the culture was removed to another tube. This gives a culture free from precipitate and stringy growth clumps. To further remove, as far as possible, any remaining clumps the culture was filtered through sterile glass wool. This procedure was followed in preparing all cultures for inoculation.

2. Birds Used

The birds used in these inoculation experiments were from various sources. All were in good physical condition except some birds lacking vitamin A. These latter birds had been on experiment approximately nine weeks and were just beginning to show symptoms of the lack of vitamin A. On autopsy all showed slight deposits of urates in the ureters. There was also paleness of the kidneys. All

other birds used were in good physical condition and had received an adequate diet.

Some birds from both groups, i.e., the adequate diet group and those on inadequate diet, had received several vaccinations with killed cultures of Pasteurella avicida before they were inoculated with the cultures. These vaccinations had been at various intervals and with varying amounts. None of the birds showed any ill effects from these treatments. One phase of this problem was to study the development of the organism in the three groups of birds.

(1). Vaccinated.

(2). Unvaccinated.

(3). Vitamin A deficient.

In order to determine the presence of antibodies the blood of the birds was examined by means of the complement fixation test.

The standard complement fixation test as recommended by Kolmer (13) was used. All antigens used were prepared the same as Bushnell and Hudson (14) described in the preparation of Salmonella pullorum antigens for complement fixation tests.

The results of these tests were used as the basis for classifying the birds in positive and negative groups. Only birds showing fixation of complement were included in

the positive group. Vaccinated birds that did not fix complement were classified as negative.

Birds classed as vitamin A deficient were birds from vitamin A deficient pens and could be classed as such only according to the feeding record, as no test could be made to determine if the birds were suffering from lack of this vitamin except on autopsy.

3. Method of Inoculation

Inoculation was made by use of sterile 5 cc Luer syringes and 27 gauge needles. The point selected for inoculation was the most prominent vein of the fore-arm, the Vena radialis profunda. This vein is of sufficient size and capacity to be used for inoculation without danger of hemorrhage or collapse.

The vein was prepared for inoculation by first plucking all feathers in the near vicinity and washing the skin. The area was then washed with 5 percent phenol and the injection made into the vein.

The method of calculating the number of bacteria injected into the blood stream is as follows. The inoculant was plated as described, and the number of bacteria recorded. Before injection, all birds were weighed. The weight of blood in the bird was calculated as one-twelfth of the body weight and was considered as having

a specific gravity of one. From this data, the number of organisms injected into each cubic centimeter of blood could be very easily calculated. Such a method was used throughout this work.

4. Methods of Culture From Birds

Bleeding was accomplished from the same vein on the opposite wing from the one injected. The fore-arm was prepared for bleeding in the same manner as for injection except that the skin over the vein was incised for one-half to three-quarters of an inch with a sharp sterile scalpel. The vein was then punctured and the blood collected in sterile 1 percent sodium citrate in sterile 5 cc. syringes, 1 cc. of blood being collected in 1 cc. of citrate solution. This large quantity lessened the chance for error that would be incurred if smaller amounts had been collected.

The wound was then covered with sterile absorbant cotton and closely observed until bleeding had ceased. On reopening the wound for further bleeding the cotton was removed, the wound washed with 5 percent phenol and thoroughly dried. The blood flow was again initiated and from one to two cc. of blood allowed to flow before another sample was collected. This procedure gave a

sample of blood free from contaminating organisms. This technique was followed rigidly throughout this work.

Counting the number of bacteria in the recovered blood was accomplished by the plate culture method.

The diluent used was physiological saline made from c.p. salt and triple distilled water. The dilution blanks were carefully standardized and freshly made. After each dilution blank was inoculated it was agitated a definite number of times to give an even distribution of bacteria.

All pipettes were of standard size and caliberated accurately. They had been previously plugged, wrapped and sterilized.

The medium used in these plate counts differed from the medium used in growing the organism in stock and is known as hemolyzed blood agar. It consists of beef extract (Liebig's) 0.3 percent, c.p. sodium chloride 0.5 percent, peptone (Parke-Davis Bacteriologic) 2 percent, sodium citrate 0.2 percent, and agar agar 2 percent, adjusted to pH 7.2. The medium thus prepared was tubed in 9 cc. amounts and sterilized at 20 pounds pressure for 30 minutes in the autoclave. When ready for plating these tubes were melted and cooled to 45°C., then 1 cc. of hemolyzed blood was added to each tube. This hemolyzed blood was prepared by adding 10 cc. of sterile defibrinated

chicken blood to 90 cc. of sterile triple distilled water. This gave a medium highly adapted to the growth of the organism. This procedure also simplified the standardization of the amount of media added to each culture plate. It also eliminated some dangers which are encountered by using larger amounts of medium.

Throughout the plating of all blood samples the following uniform technique was observed:

(1). Accurate amounts of inoculant were added to each plate.

(2). Same temperature of medium when added to culture plate.

(3). Careful distribution of inoculant throughout the medium.

(4). Careful labeling of each plate.

Incubation of all culture plates was made in a 37°C incubator. All plates were incubated on the same level and for the same length of time, which was 24 hours.

The inoculant was plated in the same manner, in the same kind of medium and incubated the same length of time as the blood culture plates.

The surface colonies appear after 24 hours incubation as small dew-drop like colonies. Subsurface colonies are lenticular and uniform in appearence.

A Bausch and Lomb dissecting binocular facilitated the counting and the accuracy of the work by enabling the observation of any small obscure growth colonies.

5. Methods in Artificial Media

The growth of the organism was studied in artificial media. Birds that were to be used for intravenous inoculation were bled from the heart just before use. Ten cc. of blood was removed from the heart with sterile needle and syringe. Five cc. of this blood were allowed to clot and 2 cc. removed. (Labeled whole-blood).

The tubes of serum and whole blood were then inoculated with equal amounts of culture, proportional to the dosage introduced into the birds. Parallel plate cultures were made from these serum and whole blood tubes at intervals corresponding to the time of bleeding from the birds. Culturing and counting of plates was the same as previously described.

V. RESULTS OBTAINED

1. Summary of All Birds

The following table will show some of the more typical cases examined although some of the birds died too quickly to give a good growth curve. Others lived for

several days and showed considerable fluctuation in the bacterial content of the blood. Table I shows only those which died within 24 hours after inoculation.

Figure 1 shows the curve obtained from the results in Table I. This curve was obtained by plotting the log of the number of bacteria found per cc. of blood against hours of infection.

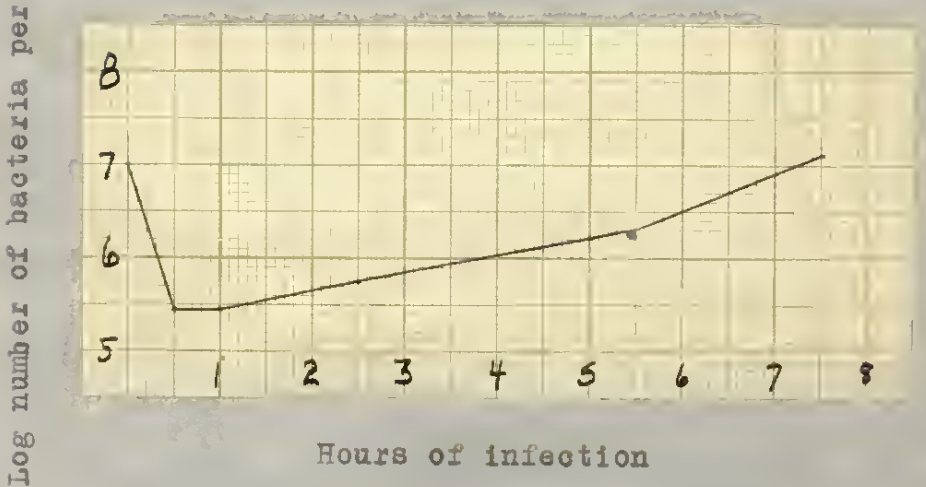

Figure 1. Average growth curve of Pasteurella avicida in all birds.

Table I. Number and bacteria per cc. in blood of birds at different intervals after inoculation.

Bird no.	No. bact. injected per cc. of body blood	Number of bacteria per cc. at intervals of				
		One-half hour	One hour	Two to three hours	Five to six hours	Seven to eight hours
3	353*	--	--	183,000,000	--	127,573*
4	471	56,200	65,000	--	3,305,000	--
5	3817	356,000	325,000	--	535,000	2,330
6	18,964	115,000	278,000	--	640,000	17,400
7	1,496	33,800	--	126,000	1,322,000	--
8	860	34,700	--	1,652,000	724,000	98,800
9	10,120	191,000	--	374,000	4,911,000	--
10	39,700	49,500	--	265,000	70,000	13,550
11	10,923	280,000	--	640,000	1,060,000	2,520
12	14,336	26,000	--	85,000	--	--
13	12,075	3,330,000	--	1,490,000	3,170,000	--

Table I. (Continued)

Bird no.	No. bact. injected per cc. of body blood	Number of bacteria per cc. at intervals of: One-half hour	One hour	Two to three hours	Five to six hours	Seven to eight hours
14	10,156	240,000	--	554,000	16,000,000	--
15	9,382	115,000	--	1,050,000	6,400,000	--
16	7,183	66,000	--	1,051,000	12,780,000	--
17	9,000	102,000	150,000	350,000	360,000	--
18	5,071	90,000	4,800,000	220,000	--	47,000
19	4,400	3,900,000	900	11,000,000	--	24,000
20	51,350	8,000	--	4,600	42,000	2,440
21	14,046	--	--	3,600	50,000	1,820
22	16,600	8,900	1,400	1,700	90,000	740
23	13,375	8,500	7,700	9,100	210,000	19,000
24	748	110,000	270,000	--	3,650,000	--
25	765	10,000	5,800	10,700	10,000,000	--
50	18,000	10,800	--	10,900	50,900	700

Table I. (Continued)

Bird no.	No. bact. injected per cc. of body blood	Number of bacteria per cc. at intervals of: One-half hour	One hour	Two to three hours	Five to six hours	Seven to eight hours
27	12,800	2,700	—	7,100	230,000	830
28	1,367	60,000	5,500	6,900	80,000	100
29	1,440	6,400	1,700	2,600	70,000	340
30	586	140,000	130,000	110,000	1,530,000	2,400
31	606	4,400	3,800	2,400	11,200	100
32	1,580	20,500	21,400	12,800	140,000	10,000
33	1,302	4,900	2,800	4,900	230,000	105,000
34	14,800	1,600	500	400	2,400	340
35	17,680	1,000	700	1,000	6,500	220
36	10,500	7,000	6,100	11,900	760,000	3,200
37	11,393	3,000	900	2,600	140,000	540
38	7,111	22,800	3,900	2,100	1,800	150
39	4,900	10,000	30,000	20,400	180,000	4,950

Table I. (Continued)

Bird no.	No. bact. injected per cc. of body blood	Number of bacteria per cc. at intervals of: One-half hour	One hour	Two to three hours	Five to six hours	Seven to eight hours
40	3765	—	—	300	20,000	440
Average	10,276	272,554	277,677	519,927	2,022,855	13,852
Log of Average	7.02	5.43	5.44	5.71	6.30	7.13

* 000 omitted in column.

It will be noted in Figure 1 that, from the time of injection into the blood stream to one-half hour after injection, an enormous decrease in the number of bacteria in the circulation had taken place. An examination of the graphs prepared from the data on individual birds shows this same decrease. In very few cases did the decrease continue for more than one-half hour.

At the end of this time the increase is greater than the decrease and the curve shows a slight upward trend. This would indicate that the remaining bacteria have become accustomed to their new environment and have assumed the normal function of reproduction. It is at this point that the aggressiveness of this organism is best shown.

How may we account for the initial drop for one-half hour? It must not be due to physical crowding or lack of nutriments but it may be due to the presence of metabolic wastes which are inhibitory to growth. The serum and whole blood apparently have no germicidial action in vitro as shown later. If the blood in vivo is germicidal the property is lost during the bleeding, defribrination, and subsequent serum removal. It is possible that the blood is germicidial only in vivo and this factor may be responsible for the first half-hour decline. Agglutination of the bacteria and subsequent removal of the

masses by filtration may offer an explanation of the removal. Heat shock, due to a change from a 98°F. habitat, which is its optimum, to a habitat of 107° F., which is considerably above optimum, may account for the death of some of the organisms. These points cannot be explained in the light of our present knowledge.

2. Summary of Each Group

A. Birds Positive to the Complement Fixation Test. In Table II is a summary of the results obtained from the birds positive to the complement fixation test. Figure 2 is the curve derived from the averages of the results obtained in Table II.

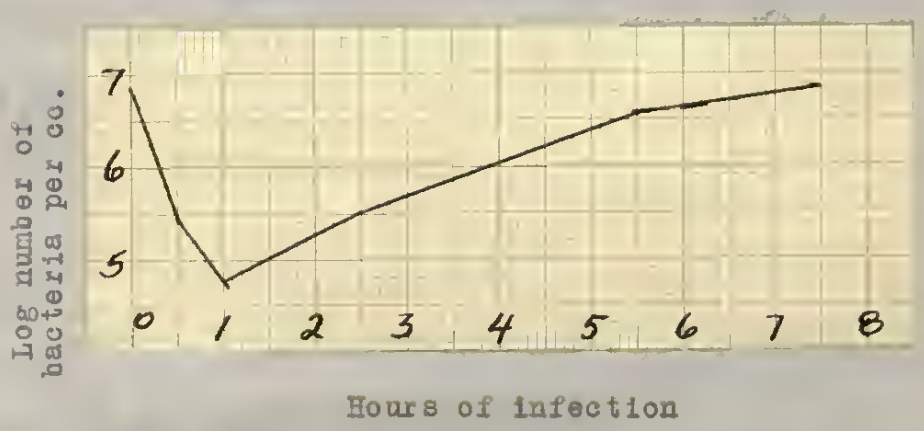

Figure 2. Growth curve of Pasteurella avicida in the body of birds positive to the complement fixation test.

Table II. Number of bacteria per cc. in the blood at different intervals after infection in birds positive to the complement fixation test.

Bird no.	No. bact. injected per cc. of body blood	Number of bacteria per cc. at intervals of					
		One-half hour	One hour	Two to three hours	Five to six hours	Seven to eight hours	
13	12,075,000	3,320,000	--	1,490,000	3,170,000	--	
14	10,156,000	240,000	--	354,000	16,000,000	--	
15	9,382,000	115,000	--	1,050,000	6,400,000	--	
16	7,183,000	66,000	--	1,051,000	12,780,000	--	
17	9,000,000	102,000	--	350,000	360,000	--	
18	8,071,000	90,000	150,000	220,000	--	47,000,000	
21	14,046,000	--	--	3,600	50,000	1,830,000	
23	13,375,000	8,500	7,700	9,100	210,000	19,000,000	
24	748,000	110,000	270,000	--	3,650,000	--	
27	12,800,000	2,700	--	7,100	230,000	830,000	
28	1,367,000	60,000	5,300	6,900	80,000	100,000	

Table II. (Continued)

Bird no.	No. bacteria injected per cc. of body blood	Number of bacteria per cc. at intervals of: One-half hour	One hour	Two to three hours	Five to six hours	Seven to eight hours
29	1,440,000	6,400	1,700	2,600	70,000	340,000
30	586,000	140,000	130,000	110,000	1,530,000	2,400,000
31	606,000	4,400	3,800	2,400	11,300	100,000
36	10,500,000	7,000	6,100	11,900	760,000	3,200,000
37	11,293,000	3,000	900	2,600	140,000	540,000
Average	7,485,000	865,000	63,944	532,000	3,300,000	7,500,000
Log of Average	6.87	5.45	4.80	5.52	6.51	6.87

On examining the curve plotted from the average of the results obtained from the birds positive to the complement fixation test it will be noted that the initial drop is lower, and continues for twice the length of time than the drop in the curve plotted for the whole group of birds. This drop is due to some unknown factor, as yet, undetermined as mentioned above.

B. Birds Negative to the Complement Fixation Test. The data collected from birds negative to the complement fixation test is presented in Table III. In the negative birds the drop continues for one-half hour which is one-half as long as the drop in the positive birds. This is followed by a sharp increase in numbers.

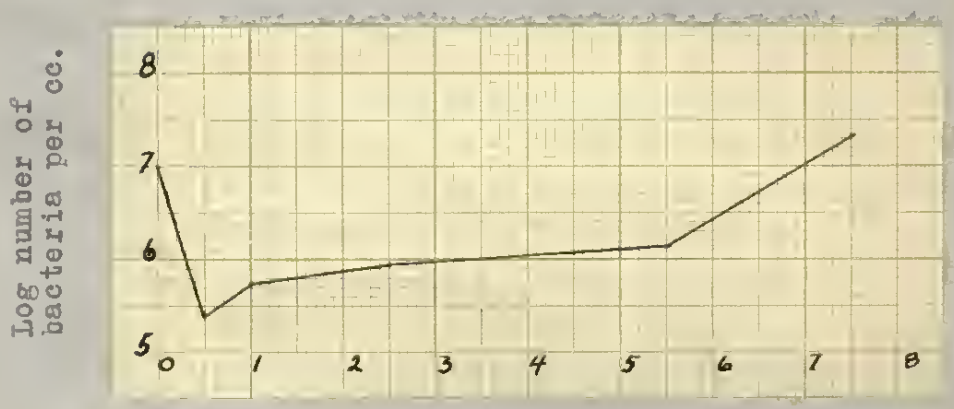

Figure 3. Curve of growth of Pasteurella avicida in the body of birds negative to the complement fixation test.

Table III. Number of bacteria per cc. in the blood at different intervals after infection in birds negative to the complement fixation test.

Bird no.	No. bact. injected per cc. of body blood	Number of bacteria per cc. at intervals of					
		One-half hour	One hour	Two to three hours	Five to six hours	Seven to eight hours	
3	35,300	--	--	183,000,000	--	127,873,000	
4	47,100	56,200	65,000	--	5,305,000	--	
5	3,817,000	356,000	325,000	--	535,000	2,330,000	
6	18,964,000	115,000	278,000	--	640,000	17,400,000	
7	1,996,000	35,800	--	126,000	1,322,000	--	
8	860,000	34,700	--	165,200	724,000	96,500	
9	10,180,000	191,000	--	374,000	4,911,000	--	
10	29,700,000	49,500	--	23,500	70,000	13,550,000	
11	10,923,000	280,000	--	640,000	1,060,000	2,520,000	
12	14,336,000	26,000	--	83,000	--	--	
19	4,400,000	3,900,000	4,800,000	11,000,000	--	24,000,000	

Table III. (Continued).

Bird no.	No. bacteria injected per cc. of body blood	Number of bacteria per cc. at intervals of: One-half hour	One hour	Two to three hours	Five to six hours	Seven to eight hours
20	51,350,000	8,000	---	4,600	4,200	2,440,000
22	16,600,000	3,900	1,400	1,700	90,000	740,000
25	765,000	10,000	5,800	10,700	10,000,000	---
26	13,000,000	10,600	---	10,900	36,900	780,000
32	1,520,000	20,500	21,400	12,800	140,000	10,000,000
33	1,507,000	4,900	2,800	4,900	250,000	105,000,000
34	14,800,000	1,600	---	---	2,400	340,000
35	17,680,000	1,000	700	1,000	6,500	220,000
Average	11,179,000	268,000	610,000	820,000	1,442,000	21,928,000
Log of average	7.04	6.42	5.78	5.91	6.15	7.34

C. Avitaminosis Birds. Table IV is a summary of the results obtained from a study of the vitamin A deficient birds used in these experiments. On autopsy all of these birds showed slight deposits of urates in the ureters and excess urates in the kidneys. These birds were from pens uniformly lacking in vitamin A and are clinically quite typical of avitaminosis A. Figure 4 was obtained by plotting the log of the averages for all the birds in this group.

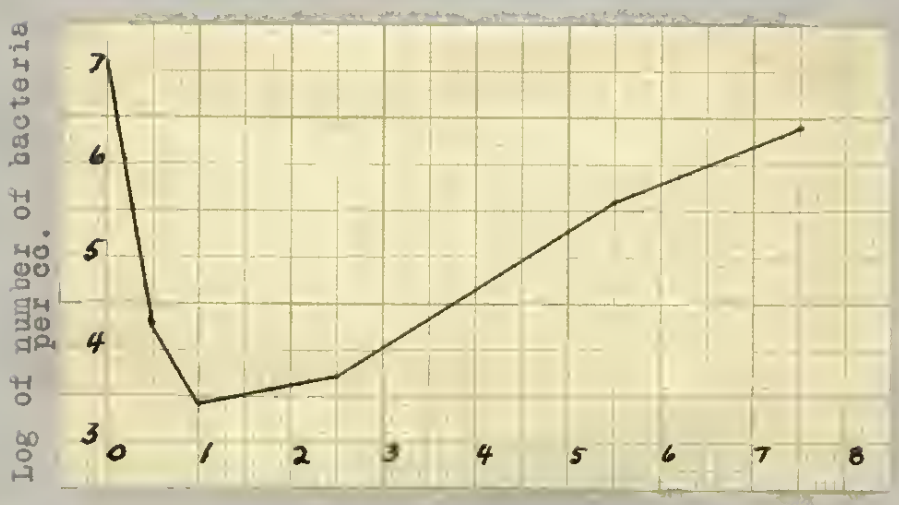

Figure 4. Graph showing growth of Pasteurella avicida in the body of birds on vitamin A deficient diet.

Table IV. Results on birds on a diet deficient in vitamin A.

Bird no.	C. bact. injected per cc. of body blood	Number of bacteria per cc. at intervals of: One-half hour	One hour	Two to three hours	Five to six hours	Seven to eight hours
28	1,367,000	60,000	5,300	6,900	80,000	100,000
29	1,440,000	6,400	1,700	2,600	70,000	340,000
26	13,000,000	10,800	—	10,900	58,900	760,000
35	17,680,000	1,000	700	1,000	6,500	220,000
34	14,800,000	1,600	500	400	2,400	340,000
30	51,350,000	8,000	900	4,600	42,000	2,444,000
25	765,000	10,000	5,800	10,700	10,000,000	—
24	748,000	110,000	—	—	3,650,000	—
21	14,046,000	—	—	3,600	50,000	1,850,000
22	16,600,000	2,900	1,400	1,700	90,000	740,000
23	13,375,000	8,500	7,700	9,100	210,000	19,000,000
Average	14,100,000	21,900	3,000	5,150	423,000	2,865,000
Log of average	7.14	4.34	3.47	3.71	5.6	6.46

3. Summary of Results in Artificial Medium

The growth curves in artificial media are presented as follows. In Table V is a summary of the results obtained from the birds used in this group. In Table VI is a summary of the growth occurring in serum obtained from the corresponding bird in Table V. Table VII is a summary of the growth occurring in the whole blood obtained from the corresponding bird in Table V. These tables are presented in graph form in Figure 5. Figure 5C is the curve showing the growth in the blood stream of the birds. Figure 5B is the growth curve of the organisms in whole blood. Figure 5A is the growth curve of the organism in the blood serum.

Table V. The rate of growth of Pasteurella avicida in the body of normal birds.

Bird no.	No. bact. injected per cc. of body blood	Number of bacteria per cc. at intervals of				
		One-half Hour	One hour	Two hours	Four hours	Eight hours
11	3,400*	80,000	47,000	2,000,000	3,500,000	1,100,000,000
918	3,900	68,000	37,000	19,000	60,000	410,000
909	--	--	--	800	27,000	230,000
14	5,400	13,800	--	6,000	40,000	420,000
24	3,000	30,000	17,100	4,500	6,800	490,000
22	2,500	--	1,400,000	900	1,100	950,000
1825	60	400	800	600	1,300	2,000
I	1,600	1,100	700	900	2,900	60,000
Average	2,900	32,216	250,000	254,000	459,000	137,820,000
Log of Average	6.46	4.50	5.39	5.40	5.66	8.14

* 000 omitted.

Table VI. The rate of growth of Pasteurella avicida in whole blood from normal birds listed in Table V.

Bird no.	No. bact. injected per cc. of body blood	Number of bacteria per cc. at intervals of					
		One-half hour	One hour	Two hours	Four hours	Eight hours	
11	3,400*	3,000*	6,000*	12,000*	78,000*	2,500,000*	
918	3,900	180	340	460	5,000	18,000	
909	--	--	--	45,000	500,000	5,600,000	
14	5,400	1,800	5,100	3,500	9,000	368,000	
24	3,000	330	280	270	4,000	800,000	
22	2,500	--	2,000	10,000	4,000,000	7,000,000	
1823	60	760	1,460	3,000	50,000	1,500,000	
I	1,600	1,040	720	1,400	700,000	--	
Average	2,900	1,185	1,990	9.452	668,000	2,255,143	
Log of average	6.46	6.07	6.29	6.97	8.82	9.35	

* 000 omitted.

Table VII. The rate of growth of Pasteurella avicida in the blood serum from normal birds listed in Table V.

Bird no.	No. bact. injected per cc. of body blood	Number of bacteria per cc. at intervals of					
		One-half hour	One hour	Two hours	Four hours	Eight hours	
11	3,400*	5,000*	6,000*	11,000*	139,000*	2,700,000*	
918	3,900	28,000	46,000	58,000	98,000	3,400,000	
909	--	--	--	87,000	1,000,000	2,100,000	
24	3,000	420	230	320	6,000	1,200,000	
22	2,500	--	3,000,000	900,000	300,000	1,900,000	
1823	60	870	1,050	4,000	60,000	600,000	
I	1,600	24,000	30,000	53,000	1,000,000	2,300,000	
Average		11,658	13,380	161,900	372,000	2,030,000	
Log of average	6.46	7.06	7.12	8.20	8.57	9.30	

*000 omitted.

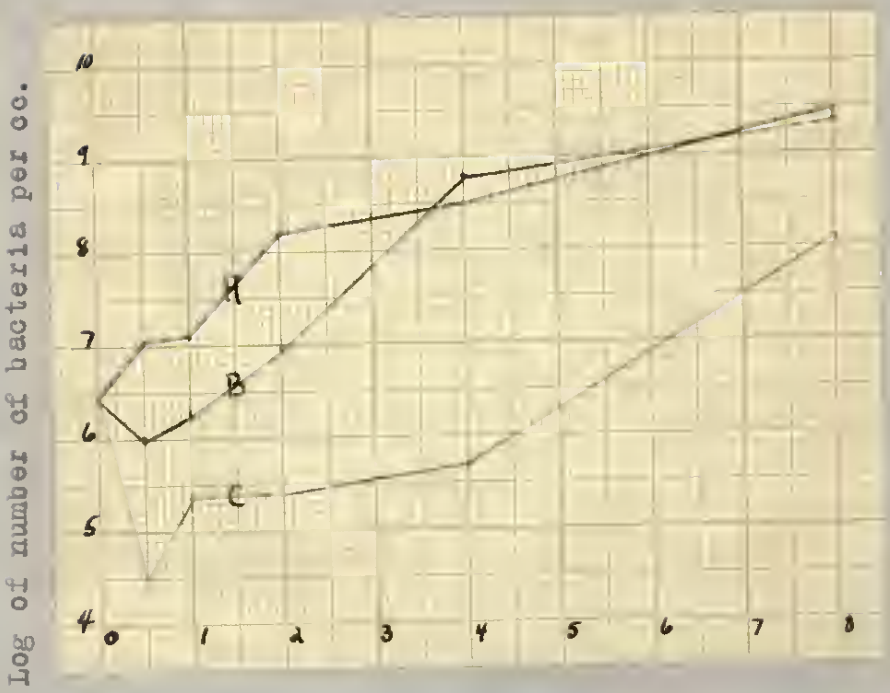

Figure 5. The growth curve of Pasteurella avicida in: (C)- the body of normal birds; (B)- whole blood from the same normal birds; (A)- blood serum from the same birds.

VI. DISCUSSION OF RESULTS

In all groups of birds the symptoms of the disease on artificial infection were very similar to spontaneous field cases. Cyanosis of the comb and wattles was general and could be noticed in all groups for some time prior to death.

On autopsy, all birds were cultured from the heart

on hemolysed blood agar. The growth from the isolated culture was then transferred to dextrose, lactose, maltose and saccharose fermentation tubes. Growth was secured in all cases and Pasteurella avicida in pure cultures was isolated from all the birds autopsied. As shown by Gram's staining and formation of acid in dextrose and saccharose, all cultures were considered to be pure and typical.

Typical findings in the digestive tract are as follows and are generally true of all birds autopsied: Edema of walls of intestine and excessive fluid excretion, congestion of duodenum and hemorrhagic enteritis. The liver is swollen, pale and hemorrhages are present in the proventriculous. The digestive tract is generally congested. The lungs are very edematous and a serous exudate is present. The kidneys are usually pale and in a few cases petechial hemorrhages are found on the surface. Petechial hemorrhages on the heart are common and are present in practically all cases.

Disregarding the clinical findings of avitaminosis A, which were confined to one group of birds that were known to be in that condition, the post-mortem findings are very typical of field cases of fowl cholera. From a clinical point of view it may be stated that artifi-

cial inoculation by the intravenous route produced typical fowl cholera which does not vary from field cases of an acute nature. This conclusion is based on the course of the disease, and the pathological and bacteriological findings.

The group as a whole shows the presence of bacteria in the lungs, liver, spleen and heart blood. This was demonstrated by the use of klatsch preparations stained with methylane blue. The greatest number of bacteria were observed in the heart preparations and comparatively smaller numbers in the other organ tissue preparations. Occasionally some phagocytic action could be seen but the total amount observed was small.

On examining the curve of growth of the organisms in the blood stream of all the birds taken as a group, several general and outstanding facts will be noticed. First, there was a decided decrease in the number of organisms free in the blood stream at the end of one-half hour. Second, there was a gradual increase in the number from the end of the one-half hour period to the end of the seven and one-half hour period.

An explanation of this decrease is impossible at present. Some of the most logical theories, yet to be studied, are as follows:-

(1). Mechanical filtration.

(2). Germicidal action of blood in vivo.

(3). Heat shock.

(4). Agglutination and phyogocytosis.

It is necessary for the organism to become adapted to its new medium and the consequen development in the blood stream is necessary before the true aggressiveness of the organism is fully developed. On entering the blood stream, the organisms may find a medium which is antagonistic to their growth and may actually be killed. After a period of inhibition, which appears to end in one-half hour in most cases, the aggressive nature of the organism develops and there is a steady increase from the low point of decline to the death of the bird.

That the aggressiveness of the organism is inhibited to some extent is indicated by some studies on the generation time. That this work is liable to a great deal of error is also shown by the probable error determinations.

The generation times were calculated by the use of Muller's generation time formulae.

$$G = \frac{T \cdot \text{Log } 2}{\text{Log } B - \text{Log } A}, \text{ in which}$$

G = Generation time.

T = Elapsed time between A and B.

B = Final number of organisms.

A = Initial number of organisms.

The following results are obtained by averaging the generation times of the organisms for the whole group of birds from period to period.

The probable errors have been figured from the same data as used in finding the average generation time, i.e., for all birds for each period. The calculation of probable error was made by use of the following formula.

$$d=\frac{1}{N}\sqrt{N\cdot\Sigma X^2-(\Sigma X)^2}$$. in which

P.E. = d x 0.6745.

d = Standard deviation.

N = Number of generation times.

X = Generation time.

In Table IX an estimate, as shown by the probable error, is made of the rate of growth of the organisms in vivo at various intervals. The generation time in the one-half to one hour period, speaking empirically, is short.

Table VIII. A summary of the generation time of Pasteurella avicida when grown in the circulating blood of birds and in blood serum and whole blood outside the body.

Group	Period covered	Generation time
All birds	1 to 7½ hours	47 ± 19.5 min.
Positive to complement fixation	" " " "	60 ± 13.4 min.
Negative to complement fixation	" " " "	90 ± 49.2 min.
Vitamin A deficient	" " " "	62 ± 18.8 min.
Donors of whole blood and blood serum	" " " "	70 ± 24.9 min.
Whole blood	½ to 8 hours	41 minutes
Blood serum	" " " "	60 minutes

The generation time with the probable error for each period, using the average of the whole group of birds, is presented in Table IX.

Table IV. The generation time and probable error of the whole group of birds from period to period.

Period	Generation time
½ to 1	24 ± 7.45 minutes
1 to 2½	108 ± 28.75 minutes
2½ to 5½	74 ± 24.28 minutes
5½ to 7½	36 ± 14.83 minutes

Assuming that the medium in which the organisms have been placed is injurious, it destroys some of the organisms and the subsequent high generation time in the one to two and one-half hour period results. The generation time in the two and one-half to five and one-half hour period shows a decidedly lower figure. The organism must have become adapted to its new habitat and is again reproducing normally in its role as an aggressive organism. This is substantiated by the figure for the generation time of the five and one-half to seven and one-half hour period which approaches near the generation time of the one-half to one hour period.

Using the same data as used for figuring the period generation time and probable errors the following cal-

culations were made. The generation time on the whole group of birds, using the generation time for each bird, for the one to seven and one-half hour period is 47 ± 19.5 minutes. These figures show the wide variation that is to be expected in this type of experiment. Calculating the average generation time for the organism by using the average generation time for each period the following figures were obtained: 50 ± 20.9 minutes.

Results from birds positive to the complement fixation test are presented in Table II. The results are summarized in Figure 2. Several facts are outstanding and are to be noticed in this group of birds. First, the period of decline continues to the end of one hour. This period is twice as long as the period of decline in the group as a whole. This may be due to the agglutination of the bacteria in a positive serum, that is, specific agglutination. If this is true, the bringing together, or agglutination of bacteria into larger clumps, would enhance the removal by filtration and phagocytosis. The longer period of decrease is also accompanied by a proportionally greater quantitative decrease than is incurred in the group as a whole. Actually a positive serum will agglutinate some organisms <u>in vivo</u>. Theoretically, that may account for the

decrease in the number of organisms in the first hour after injection into the blood stream of the bird. In reality, we do not know whether agglutination takes place or not. The end point of the growth curve in the group of birds positive to the complement fixation test is slightly lower than the end point of the group as a whole.

The data presented in Table III and summarized in Figure 3 was obtained from birds negative to the complement fixation test. It will be noted that the curve in Figure 3 compares very closely to that in Figure 1, which was prepared mostly from normal birds. It will also be noted that the end point of the curve is slightly higher than the end point of the growth curve obtained from positive birds. This is the variation that would be expected between the growth curves of bacteria in the blood stream of positive and negative animals.

The results obtained on the group of birds deficient in vitamin A are summarized in Table IV and Figure 4. This presents a growth curve that differs from the growth curve of the organism in any other group of birds. The length of time of decrease is comparable to the one hour period in the growth curve

of the organism in the body of birds positive to the complement fixation test. To account for this long continued decrease several theories have been suggested.

(1). Nutritional deficiency.

(2). Congestions of organ tissues.

(3). Presence of an excess of urates in blood and tissues.

Tables V, VI and VII contain summaries of the experimental work conducted on artificial media. The generation time shown in Table VIII in which the parallel determinations were made in the bird the serum and whole blood tube, is 75 ± 22.9 minutes. This is for the one-half to seven and one-half hour period. The generation time for the culture, in whole blood, is 41 minutes, and for the culture in serum, 60 minutes.

Upon examination of Figures 5A, 5B and 5C it will be noted that the growth curve for the organism *in vivo* assumes practically the same proportions as the whole group growth curve, after the initial drop. The curve representing the growth in whole blood shows a marked drop at the end of the one-half hour period. This decrease in numbers may be caused by several factors.

(1). Phagocytic action.

(2). Germicidal action of the whole blood.

(3). Natural death due to an unfavorable medium. As yet, we are unable to state the true cause of this decrease.

The curve of growth, of the organisms in serum, fails to show the drop at the end of the one-half hour period, as it does in the whole blood. Instead, it shows a marked rise. This might be explained by the statement that the serum was cell free, more favorable for the growth of the organism and non-germicidal. How true this statement is must yet be determined.

From the one-half hour period to the end of eight hours, the curves representing the growth of Pasteurella avicida in artificial medium do not differ materially from the growth curves of organisms in vivo and vice versa.

VII. CONCLUSIONS

It will be difficult to draw many definite conclusions from this work. Some general conceptions which have as yet to be proved may be presented. Doubtless it can be stated that when Pasteurella avicida is injected intravenously in large doses into the blood stream of domesticated fowl, a drop in the number circulating in the blood stream occurs. This drop cannot

be definitely accounted for. It may be due to:

(1). Filtration.

(2). Germicidal action of blood.

(3). Lose of virulence, or aggressiveness.

That the drop in numbers is not due to heat shock is indicated by the following investigation.

Two tubes of whole blood were inoculated with equal amounts of culture. One was incubated at 37° C and the other at 41.7°C, which was to approach the body temperature of a bird. Plate counts from these tubes at stated periods indicate no drop due to heat shock. Table X below shows the results obtained from this experiment.

Table X. The growth of Pasteurella avicida at 41.7°C and at 37°C.

Temperature	Hours after inoculation					
	* 0	½	1	2	4	8
41.7°	8.15	8.19	8.69	9.44	9.47	9.54
37°	8.10	8.13	8.58	9.32	9.52	9.87

*Bacteria per cc.

It was considered doubtful that the 5° sudden change in temperature would be destructive to any bacteria. It was considered worthy of study, however. This temperature change does not have any lethal effect on the organisms as

indicated by the log of the averages for each period in Table X. Growth occurs normally at 41.7°C. This does not explain the death of any organism on intravenous injection into a new and higher temperature medium.

Fowl cholera may be produced experimentally by intravenous injections of Pasteurella avicida. The disease produced does not differ from acute field cases.

Vitamin A deficient birds do not appear to be any more susceptible to intravenous injections of Pasteurella avicida than do birds on an adequate diet.

Birds positive to the complement fixation test for Pasteurella avicida show no more added resistance to intravenous inoculation than do birds negative to the test.

The curve of growth of Pasteurella avicida in vivo is the same as that for growth under other conditions.

Intravenous injections of Pasteurella avicida produced a general septicemia throughout the body.

VIII. ACKNOWLEDGMENTS

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